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^{1}H NMR spectroscopic analysis detects metabolic disturbances in rat urine on acute exposure to heavy metal tungsten alloy based metals salt

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ABSTRACT

Heavy metal tungsten alloys (HMTAs) have been found to be safer alternatives for making military munitions. Recently, some studies demonstrating the toxic potential of HMTAs have raised concern over the safety issues, and further propose that HMTAs exposure may lead to physiological disturbances as well. To look for the systemic effect of acute toxicity of HMTA based metals salt, ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopic profiling of rat urine was carried out. Male Sprague Dawley rats were administered (intraperitoneal) low and high dose of mixture of HMTA based metals salt and NMR spectroscopy was carried out in urine samples collected at 8, 24, 72 and 120 h post dosing (p.d.). Serum biochemical parameters and liver histopathology were also conducted. The ^{1}H NMR spectra were analysed using multivariate analysis techniques to show the time- and dose-dependent biochemical variations in post HMTA based metals salt exposure. Urine metabolomic analysis showed changes associated with energy metabolism, amino acids, N-methyl nicotinamide, membrane and gut flora metabolites. Multivariate analysis showed maximum variation with best classification of control and treated groups at 24 h p.d. At the end of the study, for the low dose group most of the changes at metabolite level reverted to control except for the energy metabolites; whereas, in the high dose group some of the changes still persisted. The observations were well correlated with histopathological and serum biochemical parameters. Further, metabolic pathway analysis clarified that amongst all the metabolic pathways analysed, tricarboxylic acid cycle was most affected at all the time points indicating a switchover in energy metabolism from aerobic to anaerobic. These results suggest that exposure of rats to acute doses of HMTA based metals salt disrupts physiological metabolism with moderate injury to the liver, which might indirectly result from heavy metals induced oxidative stress.

1. Introduction

The use of depleted uranium (DU) as an anti-armor ballistic material has raised several controversial issues for the environment and health related problems. The toxic effects of DU, which came into sight during its use in the Gulf war and the war in the Balkans have promoted the investigations of other material such as heavy metal tungsten alloys (HMTAs) as non-toxic and safer alternative to DU. HMTAs are dense heavy metals composed of a mixture of tungsten (W 91–93%), nickel (Ni 3–5%) and either cobalt (Co 2–4%) or iron (Fe 2–4%) particles, which are increasingly being adopted as the raw material to make parts of military products. Due to impressive ballistic properties of HMTAs such as high density, strength, hardness, and relatively inert nature of tungsten and its alloys, HMTAs have replaced DU in kinetic energy penetrators and lead (Pb) in some small caliber ammunition [1,2].

A study by Kalinich et al. [3] has raised several questions regarding safety issues and health effects of HMTAs when the pellets containing 91.1% W, 6% Ni, and 2.9% Co showed rapidly developing aggressive metastatic tumours in rats at the implantation site. This unanticipated finding raised extreme concern over the

Abbreviations: HMTA, heavy metal tungsten alloy; BW, body weight; D_2O, deuterium oxide; DU, depleted uranium; ROS, reactive oxygen species; ^{1}H NMR, ^{1}H nuclear magnetic resonance; TSP, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid; MSDS, material safety data sheet; FIDs, free induction decays; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LD_{50}, half-lethal doses; SD, standard deviation; p.d., post dose; i.p., intraperitonealy; NOESYPR1D, nuclear overhauser enhanced spectroscopy; PR, pattern recognition; PCA, principal component analysis; PLS-DA, partial least square-discriminant analysis; MANOVA, multivariate analysis of variance; RBC, red blood cells; NAG, N-acetylglutamate; NMN, N-methyl nicotinamide; TCA, tricarboxylic acid.

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potential health effects of HMTA-based munitions and also due to long-term exposure from internalized retained HMTA fragments in military and civilian victims; it became a major focus of attention by the military medical community [2,4,5]. Some studies have also demonstrated the carcinogenic potential of HMTAs, and exhibited a synergistic effect that exceeded the effects of individual metals in the form of malignant transformation, generation of reactive oxygen species (ROS), oxidative DNA damage, and expression of several stress genes in cultured cells when exposed to military relevant mixture of W, Ni and Co [6–8]. These studies suggest the potential toxicity of HMTAs, which needs to be evaluated in detail. Though several studies have demonstrated carcinogenic and epigenetic changes induced by HMTAs, studies related to the physiological perturbations due to acute toxicity of HMTAs are still limited. Therefore, there is a need to investigate the pathophysiological perturbations associated with the toxicity of HMTAs, which in turn may facilitate identification of HMTAs toxicity associated health effects.

NMR spectroscopic analysis coupled with multivariate statistical chemometric methods is a powerful approach to assess the toxicological effects of environmental toxins. Metabolomics [9] illustrate the non-biased analysis of molecules present in complex mixtures, which can be used to identify biomarkers specific to certain stimuli. Metabolomics have the potential to evaluate the metabolic changes in response to drugs, environmental stress or diseases, and could help in improved diagnosis as well as treatment [10–12]. In recent years, NMR based metabolomics have been widely used for assessment of toxic mechanisms, prediction of toxicity and identification of many organ based metabolite markers associated with heavy metal induced toxicity [13–20]. The metals used in HMTAs have both chronic and acute effects on the cellular processes [21–24]. However, the information on the toxic effects of these metals together in military relevant proportions at the metabolite level is inadequate. Therefore, the present study has been designed to investigate the effect of acute exposure of HMTA based metals salt in rat urine using NMR spectroscopy in conjunction with multivariate data and pathway analysis.

2. Materials and methods

2.1. Chemicals

All chemicals, NMR solvents, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) and deuterium oxide (D_2O), $Na_2WO_4{\cdot}2H_2O$, $NiCl_2$ and $CoCl_2$ used in the study were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Dose preparation

The stock solutions of all the three metals salt viz. $Na_2WO_4{\cdot}2H_2O$, $NiCl_2$ and $CoCl_2$ were prepared separately in 0.9% saline on the basis of material safety data sheet (MSDS) information as reported earlier [25–27]. Since all three salts had to be mixed in proportion to military relevant mixture (91:5:4). Therefore, a fixed volume of 500 μl of dose (both low and high) comprising 465, 25 and 10 μl of tungsten, nickel and cobalt salt, respectively was prepared from their respective stock solution and injected intraperitonealy (i.p.) in rat. The concentrations of the stock solutions of $Na_2WO_4{\cdot}2H_2O$, $NiCl_2$ and $CoCl_2$ were 25, 25 and 50 mg/ml, for low dose injection and for high dose injection the concentrations of the stock solutions were varied to 75, 75 and 150 mg/ml, respectively. The low dose of metals salt was in the range of one tenth to one fifth of LD_{50} per kg body weight. In case of the high dose, it was between two fifth and four fifth of LD_{50} per kg body weight.

2.3. Animal handling and treatment

A total of 66 male Sprague Dawley rats, 11 weeks of age (225 ± 5 g) obtained from the animal facility of the institute were acclimatized for 7 days in polypropylene cages at room temperature, 22 ± 2 °C, and relative humidity at a level of 50 ± 10%. The light cycle was maintained at 12 h of light and 12 h of darkness. Food and water were provided *ad libitum*. Rats were randomly divided into three groups (control, low dose and high dose). Two groups with an equal number of animals ($n = 24$ in each group) were injected i.p. with low and high dose of a military relevant mixture of HMTA based metals salt. Control rats ($n = 18$) were injected with 0.9% saline (i.p.). For urine collection, animals ($n = 6$ in each group) were randomly picked from all the three groups and were kept in metabolic cages for acclimatization for 3 days. All animal handling and experimental protocols were performed in strict accordance with the guidelines of the Institutional Animal Ethics Committee.

2.4. Body weight measurement

The body weight of each rat was measured daily between 0900 and 1000 h throughout the experimental period. The change in the body weight at 24, 72 and 120 h p.d. was compared with that of pre-treatment level for the respective groups (control, low dose and high dose).

2.5. Collection of urine, serum and tissue

Urine samples from both the dose group ($n = 6$ in each group at each time point) were collected in ice-cooled tubes containing 1% sodium azide at 8, 24, 72 and 120 h post dose (p.d.) whereas, for control ($n = 6$) urine samples were collected only once throughout the study. Supernatant was obtained by centrifugation, and then stored at −80 °C for NMR spectroscopic analysis. Since, urine can be readily and easily collected non invasively compared to other biofluids therefore, urine samples were also collected at 8 h p.d. to look for the early acute changes at metabolite level on exposure to HMTA based metals salt. Animals ($n = 6$ in each group at each time point including control) were sacrificed at 24, 72 and 120 h p.d. by cervical dislocation and 1 ml of blood and liver tissue was collected. The blood sample was allowed to clot for ∼30 min and serum was separated by centrifugation at 2665×*g* for 10 min. Collected serum was used for serum biochemical analysis.

2.6. Serum biochemistry and histopathology

The serum samples were analysed using a biochemical analyser (Erba chem. 5 plus V2, Daman, India) in order to test for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine. For histopathology, the largest lobe of the liver from control and both the treated groups was excised, fixed in 10% formalin, processed with standard histological protocol and cut into 4-μm serial sections using a microtome. The deparafinized sections were stained with haematoxylin and eosin for histopathological examination.

2.7. ^1H NMR spectroscopic measurement of urine

Urine samples (400 μl) were mixed with 200 μl of deuterated buffer solution (0.2 M Na_2HPO_4–0.2 M NaH_2PO_4, pH 7.4 containing 1 mM TSP prepared in D_2O) to minimize pH variation. To remove any precipitates present in the urine, each sample was centrifuged at 2600×*g* for 10 min and supernatant obtained was transferred to a 5 mm NMR tube. TSP was used as the chemical shift reference (δ 0.0). NMR spectral data were acquired on a Bruker-Av400 spec-

trometer (Bruker, Germany) with broad band observe probe operating at a frequency of 400.13 MHz at 298 K. Water signals were suppressed using the water-suppressed presaturation, nuclear overhauser enhanced spectroscopy (NOESYPR1D) pulse sequence. Typically, four dummy scans and 64 free induction decays (FID) were collected into 32 K data points over a spectral width of 6410.25 Hz with a relaxation delay of 2 s, a mixing time of 50 ms and an acquisition time of 2.5 s. The FID was weighted by an exponential function with a 0.3 Hz line broadening factor prior to Fourier transformation.

2.8. Data reduction and pattern recognition (PR) analysis of ^{1}H NMR spectra

Spectra were phased and corrected for baseline manually using TOPSPIN 2.1 (Bruker, Germany). Each urine ^{1}H NMR spectrum was segmented into 226 integrated regions of equal width (0.04 ppm), corresponding to the region δ 0.5–9.5 using AMIX software (Bruker Germany). Prior to any statistical analysis, regions between (δ 4.0–5.0) and (δ 5.0–6.0) were excluded to eliminate any spurious effects of variation in the suppression of the water resonance and cross-relaxation effects on the urea signal, respectively. Following removal of these regions, remaining regions of spectra were then normalised to the total integrated spectral area to reduce any significant concentration differences. The data set was mean centered and Pareto scaled before performing principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) processing.

In order to differentiate the distinct similarities in metabolic profiles of urine samples among all the groups, an unsupervised PR method, PCA, was conducted for data obtained from the urine samples. Based on PCA, metabolites differentiating the control groups from each of the treated groups were identified; and integrated. From the integrated data relative intensity of each of the metabolites were then calculated. Further, a supervised PR method, PLS-DA, was performed to maximize the separation of control and treated groups based on metabolites identified from PCA. Due to the small number of animals (n = 6, less than 20), leave-one-out cross validation was conducted to test the performance of PLS-DA model. Pattern recognition of NMR data, statistical and pathway analysis were performed using a web-based Metaboanalyst program [28].

2.9. Statistical analysis

The data of relative intensity of each of the metabolites and the concentration of serum enzymes were expressed as Mean ± standard deviation (SD). The statistical significance of the inter group variation was measured by multivariate analysis of variance (MANOVA). Multiple comparisons using the Bonferroni Post Hoc test were performed to evaluate the differences in various metabolites identified in the urine samples and the serum biochemical parameters. A paired t-test was performed to assess the statistical difference between the pre and post dosing mean body weight of animals. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Body weight and serum biochemical parameters

A significant decrease in the body weight was observed in animals of both the treated groups at 24 h p.d. whereas no change in body weight was observed in animals of the control group. At later time points i.e., at 72 p.d., the animals of the low dose treatment group showed an increase in body weight compared to 24 h and reached to its predose level by the end of the study (i.e., at 120 h), whereas weight of the high dose group animals were still reduced compared to predose and control group (Fig. 1). The changes in serum biochemical parameters are presented in Table 1. At 24 h p.d. ALT, AST and creatinine were significantly increased only in high dose group compared to controls. Thereafter, all parameters reverted near to the control level.

3.2. Histopathology

The liver of rat in control group showed centrilobular region and no sign of abnormality (Fig. 2a). In contrast, the liver of low and high dose treated groups showed mild degenerative changes in terms of moderately or distinctly blurred trabecular structure of the lobules and red blood cells (RBC) in sinusoidal regions at 24 h (Fig. 2b and d). The changes observed were more prominent in the liver of animals treated with high dose of HMTA based metals salt. However, some mild changes in both the dose groups still persisted till the end of the study i.e., 120 h (Fig. 2c and e).

3.3. ^{1}H NMR spectroscopy and PCA and PLS-DA analysis of rat urine

Visual inspection showed distinct spectral phenotypes, which were readily observed between ^{1}H NMR spectra of the urine samples collected from control and HMTA associated metals salt treated rats. Spectral peaks of metabolites were assigned according to Lindon et al. [29]. Comparative ^{1}H NMR spectra of urine from control, low and high dose of HMTA based metals salt at 24 and 120 h p.d. are presented in Figs. 3 and 4, respectively. The metabolites present in ^{1}H NMR spectra of urine of control and treated groups are associated with energy metabolism, gut flora metabolites, osmolytes, amino acids, N-acetylglutamate (NAG), N-methyl nicotinamide (NMN), membrane metabolite, and glycoproteins (Table 2).

PCA analysis of the full binned spectral region (0.5–9.5 ppm) showed that the score plots, of the high dose treatment and control group were clearly separated, while score plots of the low dose group was partially overlapped with controls at 8 h p.d. (Fig. 5a). At 24 and 72 h p.d., score plots of animals in both the treatment groups were well demarcated from that of the controls (Fig. 5b and c); whereas by the end point of the study i.e., at 120 h p.d., score plots of both the treatment group animals showed blended behavior and started approaching towards controls (Fig. 5d). PCA analysis showed best separation of the treated groups from controls at 24 h p.d. (Fig. 5b).

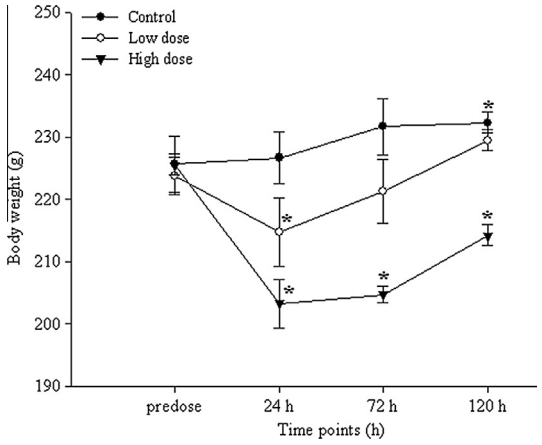

Fig. 1. Temporal changes in body weight of controls and rats exposed to low and high dose of HMTA based metals salt.

Table 1
Changes in the level of serum biochemical parameters of controls and rats treated with different doses of HMTA based metals salt. $^{*}P < 0.05$.

Parameters	Control			Low dose			High dose		
	24 h	72 h	120 h	24 h	72 h	120 h	24 h	72 h	120 h
AST (IU/L)	237.3 ± 15.2	222.4 ± 28.9	200.2 ± 11.6	295.3 ± 38.2	254.6 ± 13.5	217.8 ± 23.9	411.9 ± 63.8*	207.2 ± 14.9	231.6 ± 31.7
ALT (IU/L)	60.8 ± 8.0	49.5 ± 1.8	62.6 ± 5.1	51.3 ± 8.8	56.6 ± 12.2	59.8 ± 4.6	149.8 ± 15.2*	46.7 ± 3.9	58.0 ± 9.8
Creatinine (mg/dL)	0.77 ± 0.02	0.74 ± 0.03	0.77 ± 0.05	0.79 ± 0.03	0.75 ± 0.03	0.75 ± 0.04	0.94 ± 0.04*	0.74 ± 0.05	0.69 ± 0.06

Note: AST – aspartate aminotransferase; ALT – alanine aminotransferase.

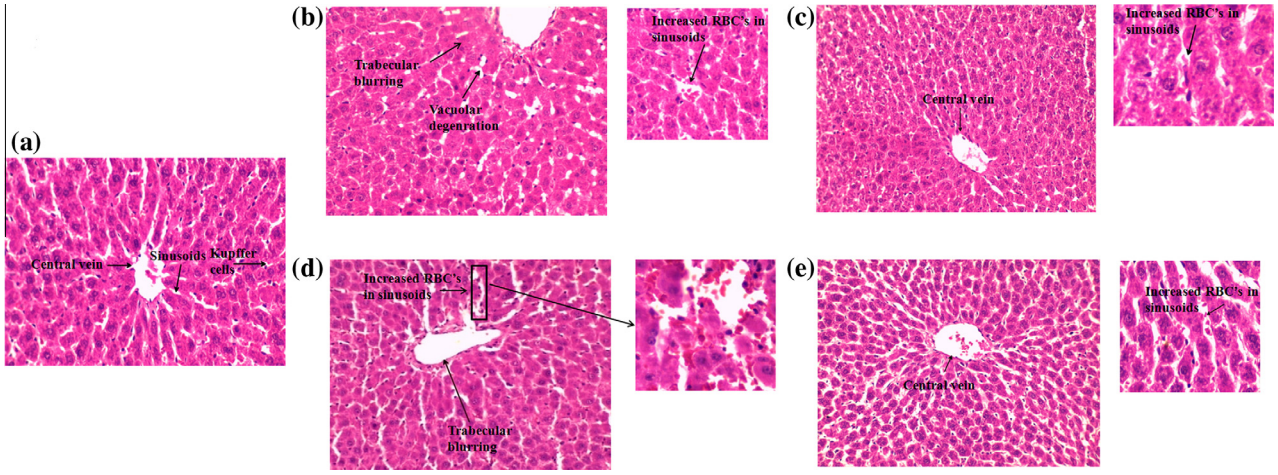

Fig. 2. Photomicrographs of liver sections with haematoxylin–eosin under light microscope. (a) Photomicrograph (200×) of control rat showing normal liver with centrilobular region. (b) Photomicrograph (200×) rats injected with low dose of HMTA based metals salt showing blurred trabecular structure of lobule, vacuolar degeneration, increased RBC in sinusoids at day 1. (d) Photomicrograph (200×) of rats injected with high dose of HMTA based metals salt showed extensive damage to liver tissue in terms of trabecular blurring and increased RBC in sinusoids (enlarged view at 400×) at day 1. (c and e) Photomicrograph (200×) of rats injected with low and high dose of HMTA based metals salt showed mild increase in RBC in sinusoids at day 5, respectively.

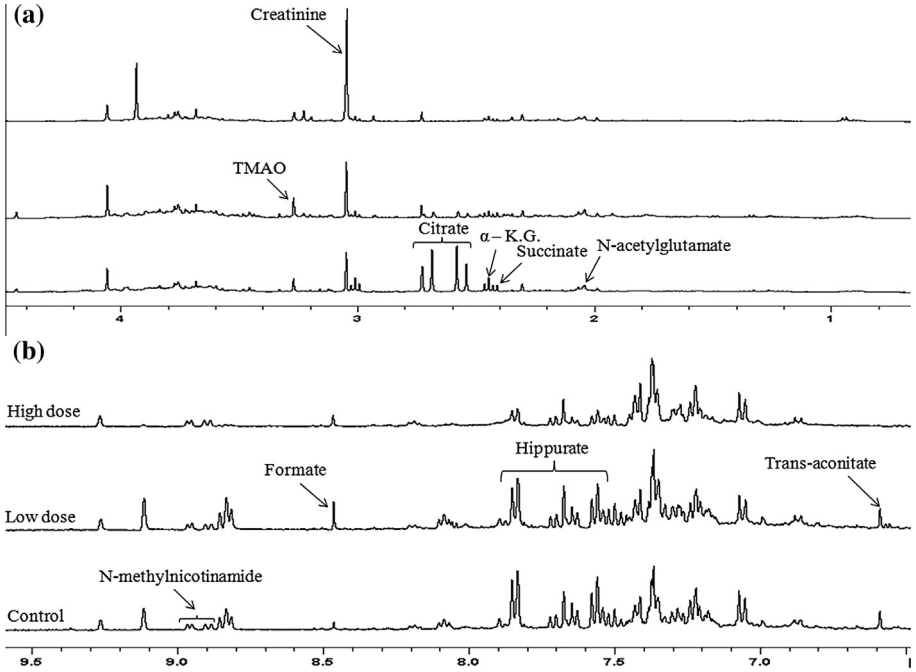

Fig. 3. Extended ^{1}H NMR spectra of urine from control and rat exposed to low and high dose of HMTA based metals salt at 24 h p.d. showing aliphatic (a) and aromatic (b) region.

In order to find out which spectral region was primarily responsible for categorization of control and treatment groups, PCA analysis of the aliphatic (0.8–3.52) and aromatic regions (6.52–9.48) was carried out separately. The PCA plot of the aliphatic binned region at 8, 24 and 72 h p.d. showed a clear separation of both the treatment groups from control group (Supplementary Fig. 1a, b,

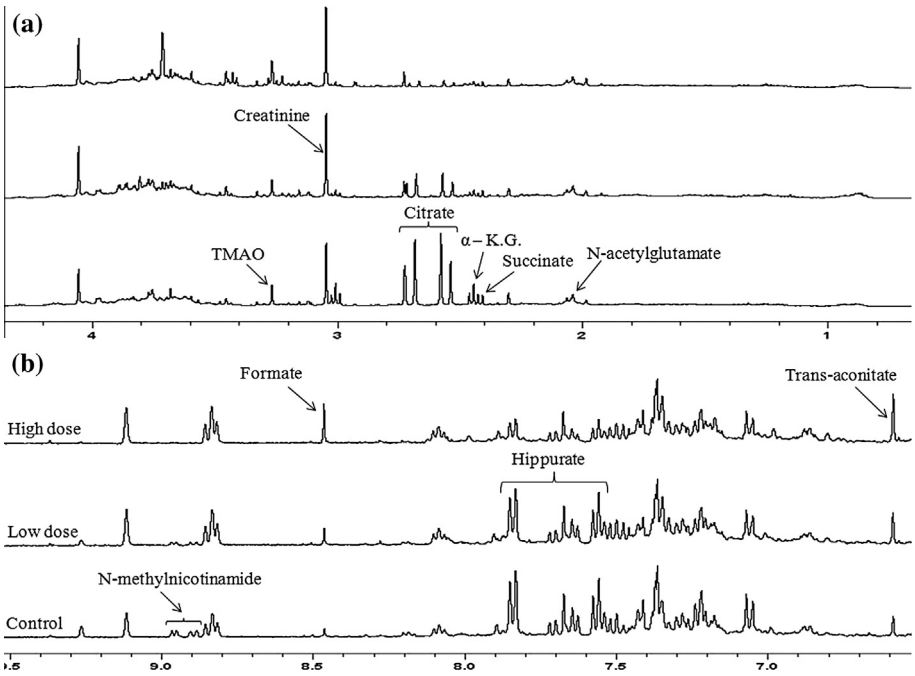

Fig. 4. Extended ^{1}H NMR spectra of urine from control and rat exposed to low and high dose of HMTA based metals salt at 120 h p.d. showing aliphatic (a) and aromatic (b) region.

Table 2
List of metabolites with their chemical shifts observed in the ^{1}H NMR spectra of rat urine of control and HMTA based metals salt treated groups.

Metabolites class	Metabolites	Characteristic chemical shifts (delta, multiplicity)
Energy metabolites	Lactate	1.33 (d), 4.12 (q)
	Succinate	2.43 (s)
	α-Ketoglutarate (α–K.G.)	2.45 (t), 3.01 (t)
	Citrate	2.55 and 2.67 (AB)
	Creatinine	3.05 (s), 4.06 (s)
	Trans aconitate	3.48 (s), 6.62 (s)
Gut flora metabolite	Hippurate	3.97 (d), 7.55 (t),7.64 (t), 7.84 (d)
Osmolytes	Trimethylamine oxide (TMAO)	3.27 (s)
	Taurine	3.25 (t), 3.43 (t)
Amino acids	Branched chain amino acids (BAA)	0.9–1.05 (m)
	Tryptophan	3.31, 3.49, 4.06 (ABX), 7.21, 7.29 (t), 7.33 (s), 7.55, 7.74 (d)
	Alanine	1.48 (d)
Membrane metabolite	Choline	3.21(s), 3.52 (m), 4.07 (m)
Glycoprotein	N-acetyl glycoprotein	2.02 (s)
Other metabolites	N-acetyl glutamate (NAG)	1.89, 2.06 & 2.1 (m)
	N-methylnicotinamide (NMN)	4.48 (s), 8.95, 8.97, 8.9 (d), 8.19 (t)
	Formate	8.46 (s)

and c). At 120 h p.d., PCA plot of the aliphatic binned region in the treatment groups were clustered around that of the control group animals exhibiting gradual recovery of animals from HMTA based metals salt exposure induced toxic effects (Supplementary Fig. 1d). In contrast, the PCA plots of the aromatic binned regions of the control and treated group animals showed blended behavior at all the time points (Supplementary Fig. 2). Therefore, it is suggested that the aliphatic regions had a major contribution in classifying the treated groups from controls.

A total of seventeen identified metabolites based on loading scores of PCA are listed in Table 2. Fig. 6 summarises the relative change in some of the observed metabolites at different post-dose time points. Further, PC loading values showed that amongst the aliphatic region, it was largely the metabolites involved in energy metabolism [citrate, α-Ketoglutarate (α-K.G.), creatinine, and succinate] that displayed maximum alterations due to HMTA associated metals salt exposure.

Additionally, a supervised analysis PLS was performed based on 17 identified metabolites. The score plots based on PLS-DA showed apparent classification as early as 8 h p.d. till the end of the study i.e., 120 h. PLS-DA showed better and clearer separation among all the three groups at all the time points compared to PCA analysis (Fig. 7). As observed in PCA, the best separation among treated and controls groups was attained at 24 h p.d. based on PLS-DA with Q^2 and R^2 values of 0.926 and 0.950, respectively.

3.4. Metabolic pathway analysis

Pathway analysis showed an impact of acute exposure of HMTA based metals salt on various metabolic pathways. For analysis, the rat (*Rattus norvegicus*) pathway library, the hypergeometric test and the out degree centrality algorithms were employed. The software provided a fit coefficient and an impact factor from pathway enrichment and pathway topology analysis, respectively. A total of 17 metabolic pathways were analysed (Fig. 8), out of which 4 metabolic pathways with 2 or 3 metabolites hits and good impact were detected at 24 h p.d. The tricarboxylic acid (TCA) cycle was identified with the highest fit coefficient value representing changes in

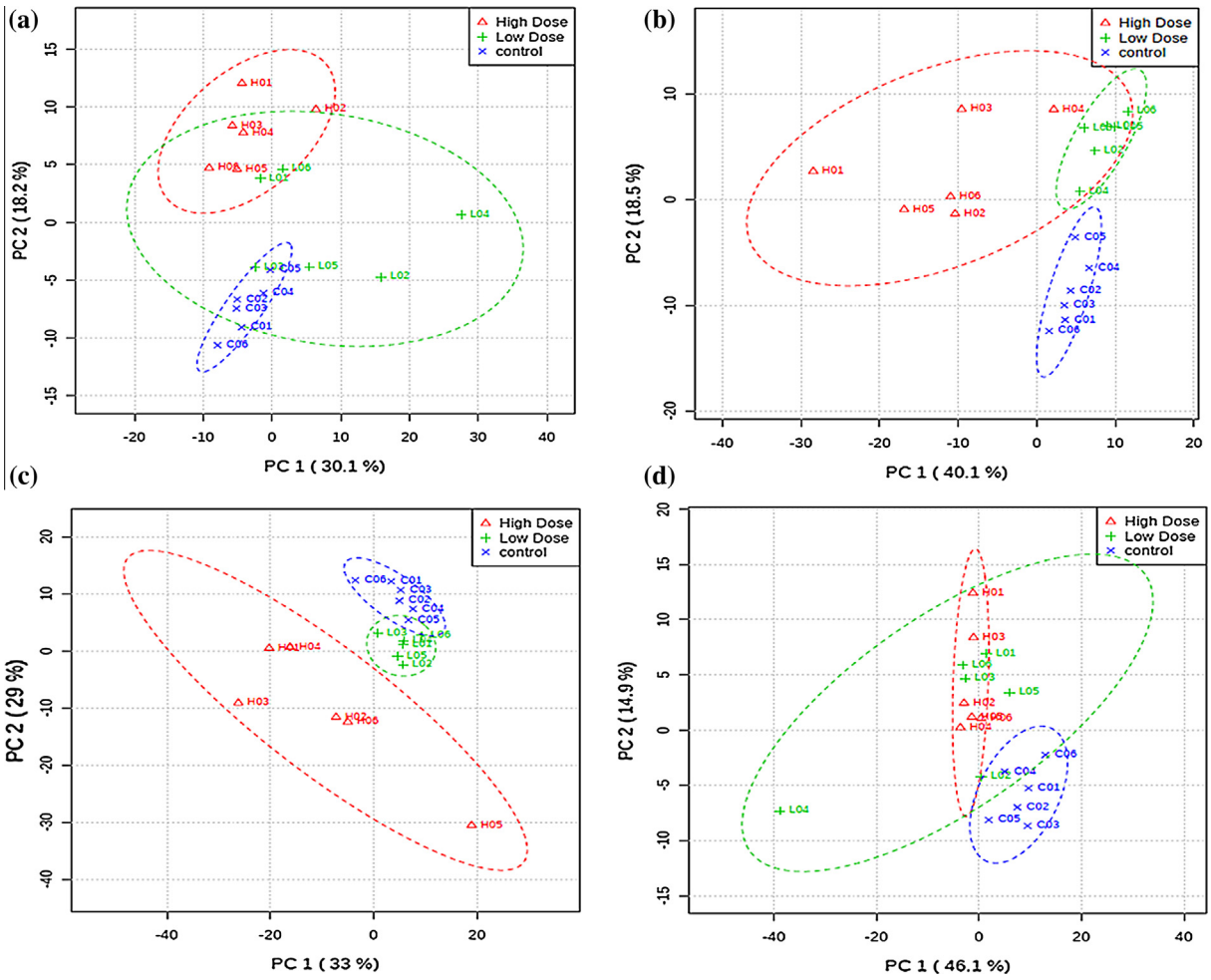

Fig. 5. The PCA score plots of full binned spectral region (0.5–9.5) at (a) 8 h (b) 24 h (c) 72 h and (d) 120 h p.d. from rats treated with HMTA based metals salt at low and high dose and control.

the cellular energy metabolism. Alanine, aspartate and glutamate metabolism, butanoate metabolism, glyoxylate and dicarboxylate metabolism were affected by acute exposure to these metals salt.

4. Discussion

The toxic effect of HMTAs in terms of carcinogenicity, genotoxicity, pulmonary toxicity and other toxic aspects have been studied but their effects at the metabolite level are still poorly understood. In the present study, an integrated NMR based metabolomic approach was used along with biochemical and histopathological methods to investigate the toxic effects of HMTA associated metals salt on physiological/biochemical processes of the body using biological fluids. In our study, both visual inspection and PCA analysis showed maximum changes related to energy metabolism in the treated groups compared to control. Metabolic pathway analysis also showed that among all the metabolic pathways analysed, the TCA cycle was the most affected at all time points indicating a switching over of energy metabolism from aerobic to anaerobic.

Heavy metals, Ni and Co associated with HMTA based metals salt, are known to cause oxidative damage to various body organs by generating ROS. The fundamental toxic mechanism of any heavy metal is the ability to form a stable complex with the sulphahydryl (SH) group of proteins, thus affecting the enzyme function. Several studies have reported decreased activity of Fe–S enzymes related to TCA and complex I of electron transport chain (ETC), increased expression of GLUT1 mRNA during Ni and Co toxicity that leads to inhibition of oxidative phosphorylation [30,31]. Though the percentage of Ni and Co was low in the HMTA based metals salt mixtures, the final concentrations of Ni and Co in the HMTA based metals salt mixture in low and high dose treatment groups were roughly one tenth of LD_{50} and two fifth of LD_{50}, respectively. Previous studies have demonstrated oxidative stress induced changes even at lower doses of Ni and Co [32,33]. It can be assumed that the changes observed in animals of both the treatment groups could be a consequence of heavy metals induced oxidative stress. Tungsten might cause disruption of cellular phosphorylation and dephosphorylation [24]. In addition, it has been reported that exposure to HMTAs with a composition of tungsten, nickel and cobalt (WNC-91-6-3), similar to that used in our study can cause significant up-regulation of genes associated with glycolysis and other enzymes involved in carbohydrate metabolism [6]. Hence, it is likely that metals associated with HMTA based salt might interfere with several Fe-dependent enzymes within the TCA cycle and electron transport chain (ETC) resulting in the diminished production of ATP in mitochondria, having significant implications in physiological perturbations. Poor ATP production due to HMTA based metals salt induced impaired oxidative metabolism affects not only cellular energy metabolism but all other pathways that require an adequate supply of ATP. The alteration in pathways namely, alanine, aspartate and glutamate metabolism, Butanoate

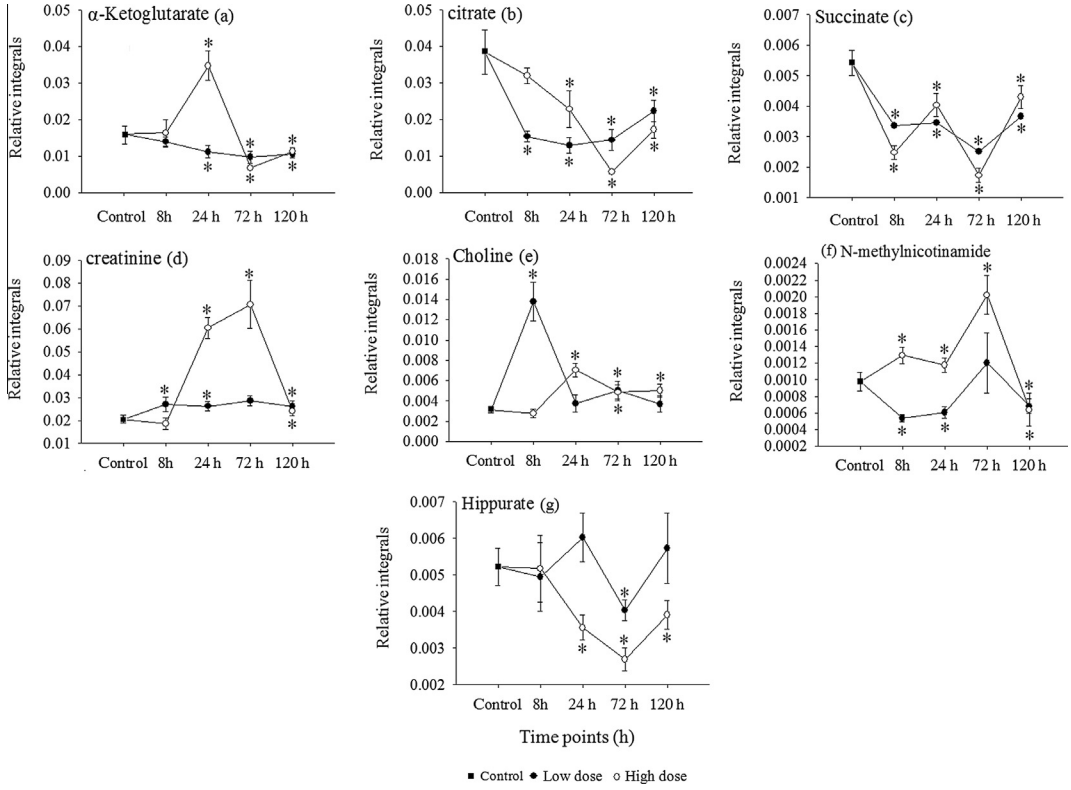

Fig. 6. Relative changes in levels of urinary metabolites (a) α-ketoglutarate, (b) citrate, (c) succinate, (d) creatinine, (e) choline, (f) N-methylnicotinamide and (g) hippurate, measured from ^{1}H NMR spectra of low and high dose of HMTA based metals salt as a function of time. Relative integral value for each of the metabolite has been shown for control animals (■).

metabolism, glyoxylate and dicarboxylate metabolism as revealed by our results on pathway analysis (Fig. 8) could also be a consequence of disturbed mitochondrial metabolism, as metabolites involved in the energy metabolism are also important intermediate of many of the above mentioned pathways.

Increased creatinine in urine spectra post dose in the treated group in our study also points towards altered energy metabolism and HMTA based metals salt induced oxidative stress. Spontaneous degradation of creatine phosphate, produced from creatine by creatine kinase, leads to the formation of creatinine which is a waste product for the body [34]. High affinity of Ni and Co to thiolate (–SH) group of creatine kinase makes it susceptible to HMTA based metals salt induced oxidative damage [35]. The inhibition of creatine kinase may increase the total amount of free creatine, which subsequently degrades to creatinine.

Another important aspect of HMTA based metals salt induced oxidative stress is disturbance of cellular integrity by the process of lipid peroxidation. Degradation of lecithin (a membrane phospholipid) results in the formation of choline via phosphocholine. Choline is further metabolised in kidney, where it gets oxidised to betaine by the enzyme choline oxidase. Ni and Co of HMTA based metals salt are known to bind to choline oxidase leading to inhibition of enzyme activity [36]. In our study, increased urinary choline level could be due to inhibition of the enzyme choline oxidase.

An indirect consequence of toxicity of metals associated with HMTA based metals salt was observed in terms of reduced urinary hippurate levels, mainly in high dose treated group. Production of hippurate requires supply of ATP via oxidative phosphorylation. Reduced production of ATP due to the impaired TCA cycle may be responsible for the decreased level of hippurate. Furthermore, decreased hippurate levels also indicate HMTA based metals salt induced toxicity to the liver as it plays an important role in the production of hippurate. The commencing step of hippurate synthesis involves binding of benzoate with coenzyme A in hepatic cells to form Benzoyl CoA by the enzyme acyl CoA synthetase. The Ni and Co might have blocked the commencing step (due to high affinity to the –SH group of the enzyme) thereby resulting in decreased production of hippurate [21–23]. In addition, the decrease in the urinary levels of hippurate might be correlated with poor intake or improper digestion of food, which is reflected by the significantly decreased body weight of the high dose group animals seen throughout the study (Fig. 1). Decreased urinary levels of hippurate have also been linked with dietary [37] and intestinal microbiota changes [38–40].

The altered levels of NMN at all time points in the treated groups compared to controls exhibited oxidative stress induced changes in the liver. NMN is a byproduct, formed during the conversion of S-adenosylmethionine (SAMe) to S-adenosylhomocysteine (SAH) and the NMN so formed is excreted in urine. The SAH is further utilized in the transsulfuration pathway to regenerate GSH. It is suggested that the increase in NMN in urine of high dose group animals might be due to the upregulation of enzyme involved in the regeneration of GSH via SAMe and other intermediates. This resulted in increased production of GSH and its utilization due to more production of ROS in high dose group as compared to the low dose, thus trying to cope up with oxidative stress.

In the present study, HMTA based metals salt induced toxicity has an indirect effect on the physiology of liver, which is well reflected in the serum biochemical parameters and histopathology. Mitochondrial metabolism and aerobic respiration are central to the physiological function of hepatocytes which is to help adjust the levels of circulating fuels and metabolites by participating in

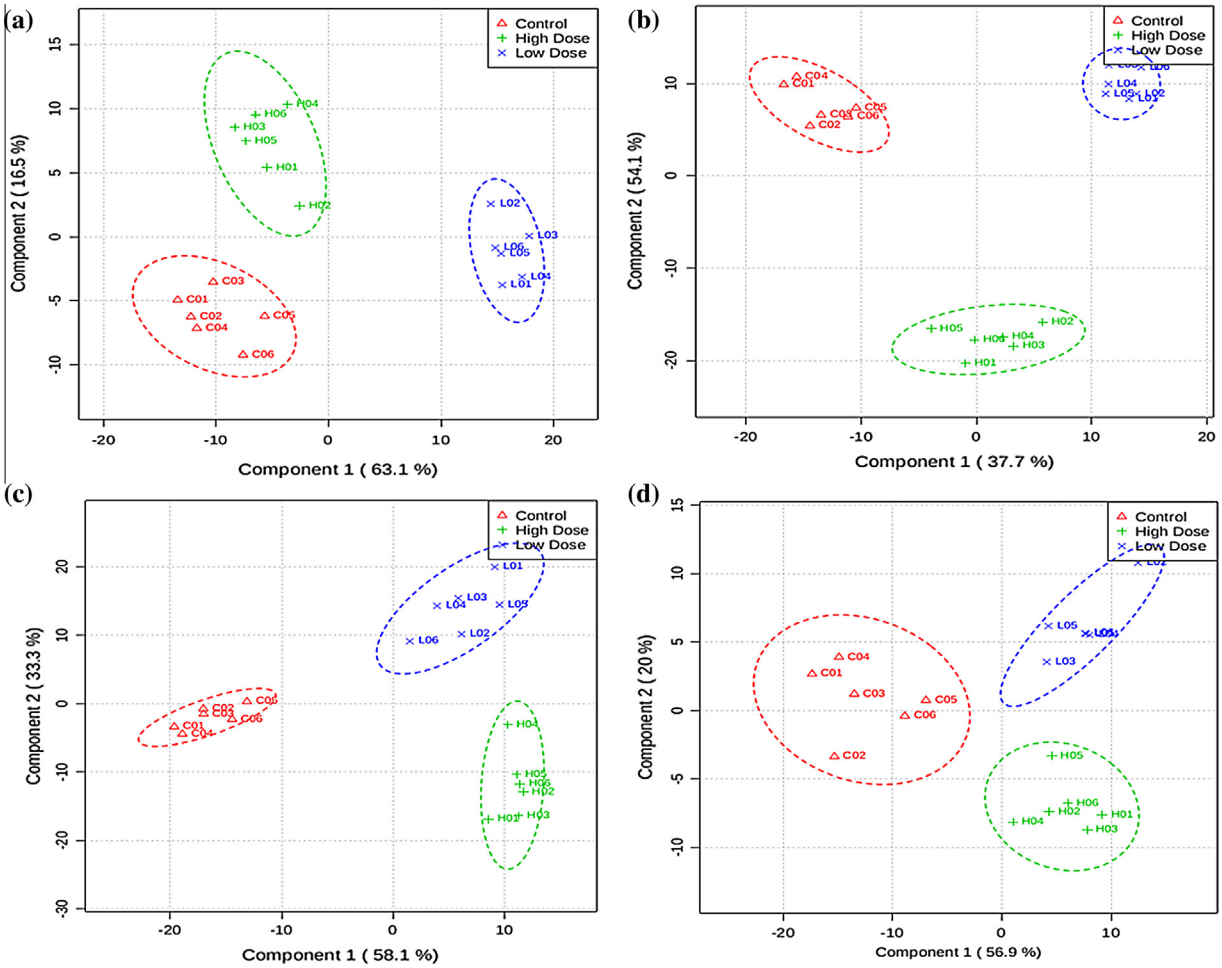

Fig. 7. PLS-DA score plot based on ^{1}H NMR spectra of urine samples at (a) 8 h (b) 24 h (c) 72 h (d)120 h from rats treated with HMTA based metals salt at low and high dose and control.

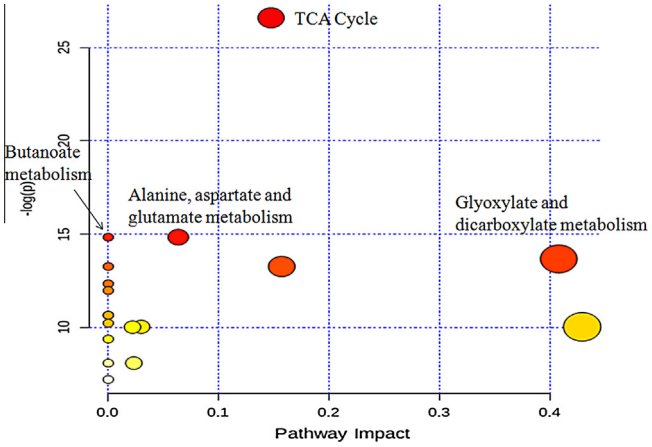

Fig. 8. Metabolite pathway mapping of the impacted metabolites identified during acute exposure of HMTA based metals salt at 24 h p.d. The analysis was performed using metaboanalyst software.

the maintenance of carbohydrate and lipid homeostasis, biosynthetic reactions, and amino acid metabolism [41]. Moreover, the liver plays an important role in the synthesis of hippurate and trimethyl amine oxide. We speculate that variation in levels of these and associated metabolites in urine spectra could be due to physiological perturbation/functional damage in the liver upon exposure to HMTA based metals salt. Most of the changes in the ^{1}H NMR spectra have been observed at 8 and 24 h p.d. with maximum changes at 24 h p.d. in both PCA and PLS-DA plots. This might be due to immediate stress induced response of the body towards toxic effects of HMTA based metals salt. At later time points i.e., at 72 and 120 h the toxic effect of metals present in HMTA based metals salt was reduced due to pharmacokinetics of metals, which is rapid and has a short retention time in the body with a half-life of 24 h [42–44]. Serum biochemical parameters reverted to normal, while mild histopathological changes still persisted. The changes in serum biochemical parameters depend upon the severity and stages of tissue damage. Histopathological changes occur at the cellular level that may be reflected in terms of altered metabolite levels in tissue extracts or biofluids, while serum biochemical parameters represent enzymatic activity associated with the organ function. There might be a chance that the subtle changes at the cellular level present at later time points may not have any effect on the enzymatic activity.

5. Conclusion

In this paper, we have demonstrated the acute toxic effect of exposure to HMTA based metals salt at the metabolic level through metabolomic approach. The toxic effect of HMTA based metals salt was observed mainly on energy metabolism and interlinked metabolic processes occurring in the body resulting in physiological disturbances in the liver. More detailed studies are warranted for a

comparative account of HMTA based metals salt and individual metal induced toxicity in order to understand the extent of damage caused by each metal component.

Conflict of interest

The authors declare no conflict of interest and competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2013.12.016.

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